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13. ABSTRACT (Maximum 200 Words) Prostate cancer (PC) cells are highly sensitive to changes in their cytoplasmic calcium and this aspect of their biology must be more thoroughly understood to uncover possible new therapeutic targets for this disease. Our preliminary studies demonstrated that calcium activated two pathways that may contribute to the calcium apoptotic sensitivity of PC cells. Calcium mediated activation of calpain results in direct activation of caspase 7 and the tyrosine phosphatase, PTP1B. To study their direct involvement in apoptosis and signaling, PC cells were transfected with dominant negative caspase 7 and inducible constructs of activated PTP1B. Dominant negative caspase 7 suppressed activation of endogenous caspase 7 by calcium ionophore, supporting a role for its recruitment into the calcium initiated apoptotic process. Activated PTP1B expression (but not a phosphatase-dead mutant) suppressed PC cell growth and blocked signaling through insulin receptor, HER2 and IL-6. However, EGFR signaling was not affected. These data demonstrate that calcium antagonizes specific signal transduction pathways important in PC cells through activation of PTP1B. Agents that regulate calcium flux and PTP1B activation in PC cells may be active in suppressing PC cells dependent on these signaling pathways for their growth and survival.				
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INTRODUCTION

Prostate cancer (PC) cells have limited sensitivity to conventional therapeutic agents due to increased apoptotic thresholds but are highly sensitivity to changes in their intracellular calcium levels. This distinction in apoptotic sensitivity may be exploited therapeutically but more information is needed to understand the respective mechanisms and processes involved. In our preliminary studies we identified two potential mediators of calcium-mediated apoptotic sensitization in PC cells. Calcium ionophore treatment of PC cells activated the calcium-sensitive protease calpain, stimulating two pathways that regulate phosphotyrosine-initiated cell signaling (PTP1B) or directly trigger apoptosis. The goal of this study is to determine the contribution of each of these processes to PC cell death and calcium flux sensitivity. The role of calpain as a master regulator of these processes is also to being investigated.

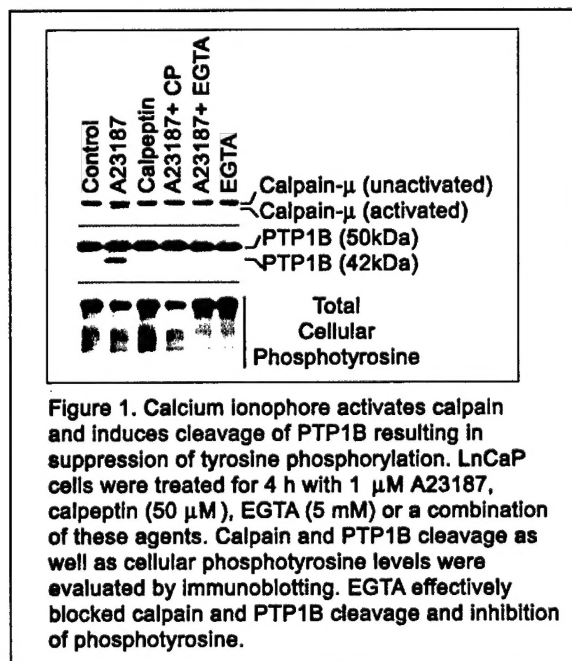
BODY

We have made great progress in completing the specific aims of this study. Listed below is an update on the progress with each aim.

Specific Aim 1. Examine the role of calpain in LnCaP cell death by inactivation of this enzyme with calpain inhibitors (chemical) or through overexpression of endogenous calpain inhibitors (calpastatin). Studies will be conducted on both wild-type and bcl-2 overexpressing LnCaP cells to determine the potential for bcl-2 to regulate calpain-mediated cell death (months 1-18).

Task 1 – measure apoptosis in calcium ionophore treated cells in the presence or absence of calpain inhibitors.

This aim has been modified since calpain inhibition by calpain inhibitors can induce apoptosis directly, possibly through effects of p53 stability. This activity complicates interpretation of the role for calpain in the activation of apoptosis or sensitization. To



compensate for this unexpected toxicity two different approaches are being used to address the role of calpain in PC cell apoptosis. 1) Cells are treated with calcium ionophores in the presence or absence of calcium chelators before calpain activation and cleavage of PTP1B was monitored. As shown in figure 1, calcium chelators block both calpain activation, PTP1B proteolysis (activation) and its cytoplasmic translocation in LnCaP PC cells, demonstrating a direct role for calcium flux in activation of calpain

and PTP1B. To confirm a role for calpain in this process we are constructing an expression vector of anti-sense calpain- μ as well as a protease-inactive (Cys to Ser mutant) mutant of this gene. This construct is being subcloned into a doxycycline-sensitive expression vector (TRex). This vector has been used successfully to induce expression of other toxic or apoptotic gene products in LnCaP cells (see below). These new approaches are being used in conjunction with acute inhibitor studies to define the role of the calcium-calpain pathway in regulation of apoptotic activity in PC cells.

Task 2 – compare in wild-type and bcl-2 overexpressing LnCaP cells.

The studies described above will also be conducted on bcl-2 overexpressing PC cells. We will also analyze effect of protease-inactive calpain- μ expression on bcl-2 overexpressing cells once construction of the expression vectors are completed.

Task 3 – transfect cells with calpastatin vectors and select stable clones expressing this protein. Measure calpain activity in calcium-ionophore treated cells, expressing calpastatin or transfected with empty vectors. Compared apoptotic sensitivity to challenge with calcium ionophores.

This aim has been modified to include tet-inducible expression of the calpastatin gene due to the toxicity of chronic calpain inhibition.

Specific aim 2.

Examine the role of caspase 7 in calcium/calpain-mediated cell death by analysis of its in vitro activation with cell lysates derived from calpain activatable or inactivatable PC cells. The role of caspase 7 in calpain-stimulated LnCaP cell death will also be assessed in vivo by measuring the effects of dominant-negative caspase 7 expression on calcium-mediated cell death (months 6-24).

Task 1 – measure caspase 7 proteolysis in calcium ionophore treated cells (in vivo). Compare in PC cells with activatable (LnCaP) and inactivatable (PC-3) calpains.

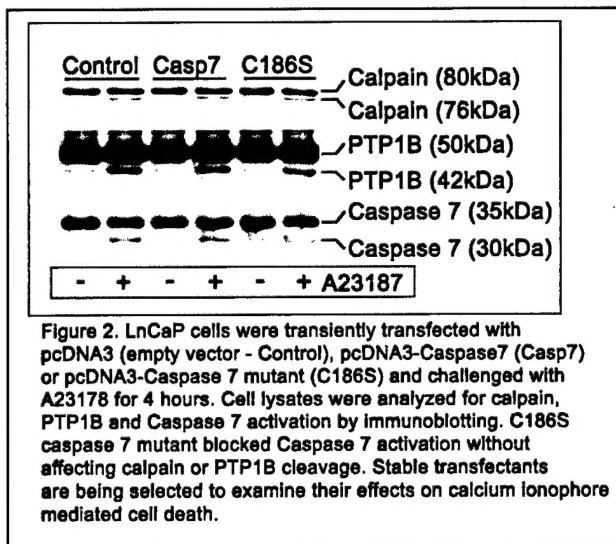
This aim is still being pursued as reported in an earlier report.

Task 2 – in vitro transcribe and translate caspase 7 and measure its proteolysis with calpain activated cell lysates. Compare with other caspases.

This aim has been completed as reported in an earlier progress report.

Task 3 – transfect LnCaP cells with caspase 7 dominant negative vector. Select transfectants positive for DN caspase 7 expression (FLAG-tagged).

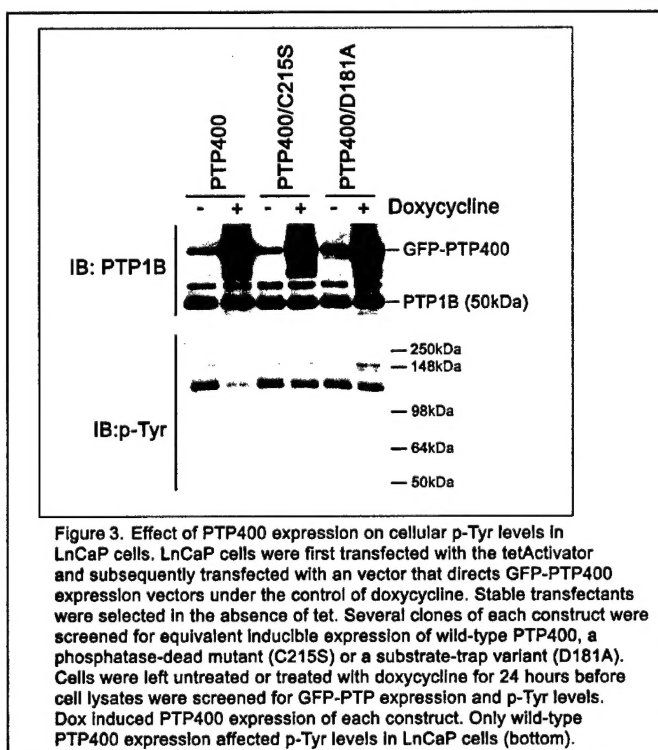
This aim is nearing completion. Dominant negative caspase 7 has been cloned and is active in partially blocking the effects of calcium ionophore on caspase 7 activation (see figure 2). Stable transfectants are currently being selected.



that encode a cytoplasmic form of PTP1B (PTP1B/400) that mimics the calpain-cleaved form of PTP1B (months 6-36).

Task 1 – transfect LnCaP and PC-3 cells with PTP1B/400. Select stable transfectants (FLAG-tagged).

Stable transfectants could not be obtained by this approach due to the effects of expression of this gene on cell cycle and apoptosis of PC cells. We chose to express PTP1B/400 under the control of a tet-activator (task 2). Much progress has been made in this aim.



Task 4 – measure cell death induced by calcium flux/calpain-activation in DN caspase 7 and vector control cells.

This aim has not yet been completed but will be tested as soon as stable DN-caspase7 clones are obtained.

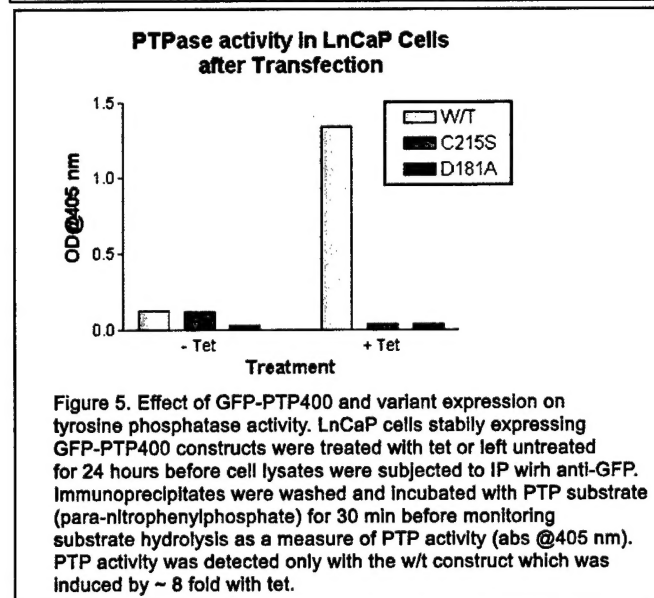
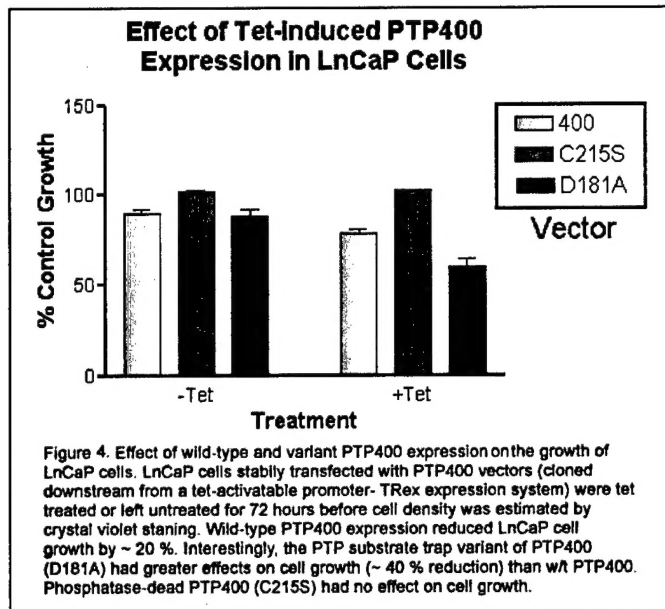
Specific aim 3.

Examine the effect of PTP1B expression on IGF-1 signaling and cell death in LnCaP and PC-3 cells using PTP1B expression vectors

Task 2 – if needed, subclone PTP1B/400 into a tetracycline-repressible vector (pEC1214). Transfect cells and select stable transfectants.

We were unable to obtain sufficient expression of PTP1B/400 using this vector. We chose to use the TRex tetracycline-activatable expression system and have obtained good results with this system (see figure 3). This construct was successfully used to monitor effects of PTP1B/400 on PC cell growth (figure 4), and PTP activity (figure 5).

Task 3 – measure effect of PTP1B expression on IGF-1 signaling by treatment with growth



factor and monitoring IRS-1 phosphorylation, MAP kinase and Akt kinase activation. Compare empty vector, tetracycline-repressed and PTP1B/400 expressing transfectants.

This task was completed using PTP1B/400 expression under the control of the tet-activator. Induced cells were challenged with growth factor and substrate phosphorylation was defined by immunoprecipitation and expression of the PTP1B/400 "substrate trap mutant". PTP1B/400 expression predominantly suppresses insulin and IL-6 signaling resulting in suppression of specific downstream cascades. PTP1B/400 blocks Stat3 activation, targeting upstream kinases that regulate these phosphoproteins (Insulin receptor, Jak2). Interestingly, insulin signaling and Akt phosphorylation were not highly affected by PTP1B/400 expression. We are now conducting studies with IGF-1 to determine whether distinctions in the role of

PTP1B/400 on insulin and IGF-1 signaling can be detected in PC cells.

To complement these studies and to provide a more rapid assessment of the role of PTP1B/400 on signaling, cell growth and apoptosis, we have completed construction of modified lentiviruses carrying the PTP1B/400 gene.

Task 4 – Measure apoptosis induced by calcium ionophore, cytokines (TNF, TRAIL) or radiation in transfectants expressing PTP1B/400 using standard apoptotic measurements.

Cell cycle changes and morphologic distinctions have already been completed. Apoptotic measures and variations in apoptotic sensitivity as well as other forms of cell death (anoikis) are currently being conducted. TRAIL receptor agonists currently in clinical trials for PC and other cancers (Human Genome

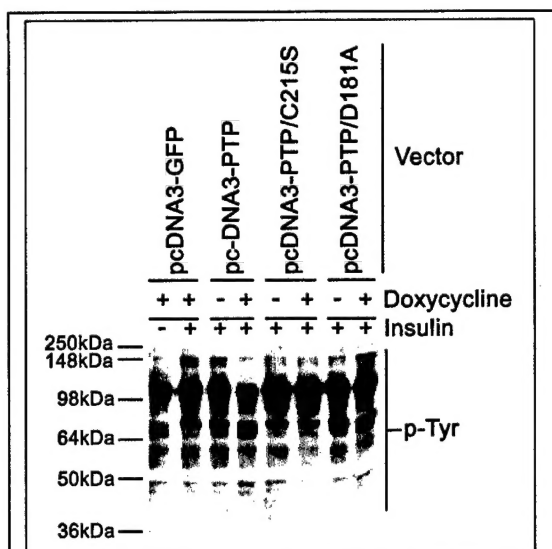


Figure 6. Effect of PTP induction on insulin-stimulated tyrosine phosphorylation. LnCaP cells stably expressing PTP400 constructs (as noted) were treated with doxycycline for 24 hours or left untreated. Cells were treated with 10 μ g/ml insulin (as noted) for 30 min before cell lysates were immunoblotted for phosphotyrosine. Only PTP400 reduced p-Tyr levels while PTP-D181A stabilized tyrosine phosphorylation of higher MW proteins.

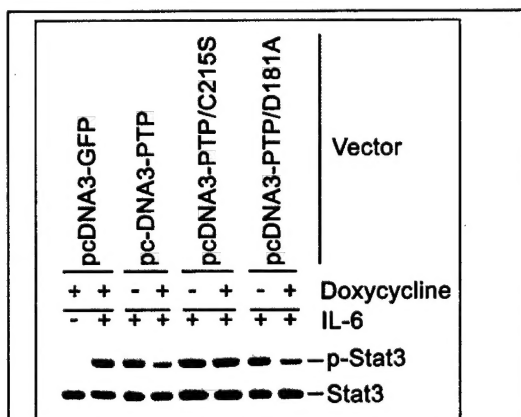


Figure 8. Effect of PTP400 expression on IL-6 mediated Stat3 activation. LnCaP cells stably expressing PTP400 constructs were treated with doxycycline (as described in figure 6) and challenged with IL-6 (10 ng/ml) for 30 min. Cell lysates were immunoblotted for phosphotyrosine-Stat3, stripped and reblotted for Stat3. Both w/t and D181A constructs of PTP400 reduced Stat3 phosphorylation.

Sciences) are being used in combination with PTP1B/400 lentiviral vectors to determine their potential to enhance cell death through activation of death receptors. Calcium ionophore and radiation-induced apoptosis will also be tested in combination with PTP1B/400 expressing cells.

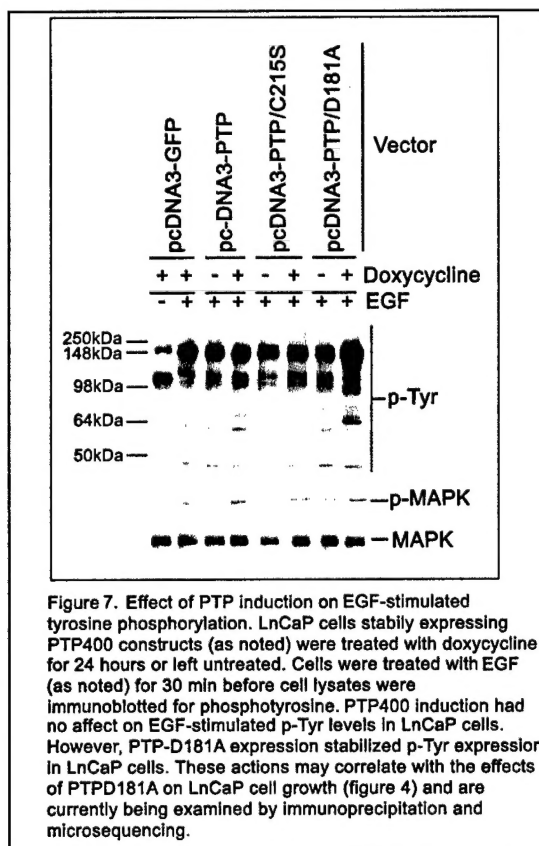


Figure 7. Effect of PTP induction on EGF-stimulated tyrosine phosphorylation. LnCaP cells stably expressing PTP400 constructs (as noted) were treated with doxycycline for 24 hours or left untreated. Cells were treated with EGF (as noted) for 30 min before cell lysates were immunoblotted for phosphotyrosine. PTP400 induction had no effect on EGF-stimulated p-Tyr levels in LnCaP cells. However, PTP-D181A expression stabilized p-Tyr expression in LnCaP cells. These actions may correlate with the effects of PTPD181A on LnCaP cell growth (figure 4) and are currently being examined by immunoprecipitation and microsequencing.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that calcium flux was responsible to calpain activation and PTP1B proteolysis.
- Established stably transfected LnCaP cells expressing the Tet activator (pcDNA6/TR transfectants; Invitrogen)). We will use these constructs to provide tet-induced expression of PTP400 (and variants) Caspase 7 dominant-negative and calpastatin.
- Demonstrated tet-activatable expression of PTP400 and variants in LnCaP cells.
- Determined that w/t PTP400 retained phosphatase activity.
- Induced PTP400 reduced insulin but not EGF stimulated tyrosine phosphorylation.
- Found PTP400 expression suppresses IL-6 mediated Stat3 activation.
- Have constructed lentiviral vectors for PTP400 cloned downstream of an IRES site and GFP expression cassette. Found high level expression in PC cells and induction of apoptosis in PC-3 cells. This system will be suitable for more in depth studies of PTP400 function in PC cells.

REPORTABLE OUTCOMES

We have generated several cell lines, constructs and vectors useful for direct assessment of the genes of interest to this project. The cell lines and vectors will also be made available to other investigators. We have not yet completed these studies but are poised to complete this project on time. We have already submitted one manuscript detailing our earlier work and have been revising the manuscript according to the reviewers comments. Acceptance is pending (J. Biol. Chem) We anticipate submission of additional manuscripts once the results are confirmed in subsequent studies. Patents for the viral vectors will also be submitted before public disclosure of the information.

One student has been granted her doctorate degree (Sharon Beresford) as a direct consequence of her involvement with this project. A technician currently working on this project has decided to apply for the Ph.D. program through our affiliated graduate program to continue his education and research on this project (Jonathan Stapley).

We have applied for additional DOD support to continue lentiviral vector studies in breast cancer. The preliminary data on PC-3 cells look very promising an NIH grant to continue this work will be submitted upon completion of this work.

CONCLUSIONS

The role of two calcium sensitive pathways in PC cell death is under investigation. These studies are focused on defining the role of calpain-sensitive proteins in this process. The difficulty exists due to the nature of the proteins under investigation since expression of both PTP1B and caspase 7 are directly detrimental to PC cells. To understand their involvement in PC cell death these proteins are being expressed or inactivated by overexpression of a dominant negative variant or tet-inducible construct. Thus far our data clearly show that

calcium flux (not calcium ionophore itself) induce calpain activation and PTP1B proteolysis, resulting in its cytoplasmic translocation. Expression of a dominant negative caspase 7 limits endogenous caspase 7 activation by calcium ionophore and further selection of stable clones will help in determining its effects on cell death. To further understand the role of PTP1B in PC cell death and signaling, a cytoplasmic form of the PTP1B protein was expressed under the control of a tetracycline promoter. Our results demonstrate that specific signaling pathways (i.e. insulin, IL-6) are inhibited by the cytoplasmic form of PTP1B (PTP400). Signal inhibition has little effect on LnCaP cell growth and responses in cells challenged with apoptotic stimuli have yet to be determined. We have been successful in generating a lentiviral vector to expedite PTP400 studies. This vector has significant apoptotic effects on PC-3 androgen independent cells but has limited effects on LnCaP cells. These results support the hypothesis of increased sensitivity of androgen independent PC cells to overexpression of PTP400. These studies are of significance since therapeutic options for androgen independent PC tumors are limited. We will be able to rapidly investigate the mechanism of action of the viral vector and will propose additional studies to determine its effects on animals bearing PC tumors. Overall, our goal of defining novel approaches to mediate PC cell growth inhibition and apoptosis are being completed through these studies. Our future goal will be to develop our basic science observations into new therapeutic agents or strategies to treat PC patients.

REFERENCES

N/A

APPENDICES

N/A